

# BACTERIAL PERMEASES<sup>1</sup>

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## I. INTRODUCTION

The selective permeation of certain molecular species across certain tissues, or into certain cells, has been recognized for a long time as a phenomenon of fundamental importance in animal physiology. The situation is, or was up to quite recently, different in the field of microbiology. Although the importance of recognizing and studying selective permeability effects had been frequently emphasized, particularly in recent years by Doudoroff (22) and by Davis (18), the available evidence appeared ambiguous, and the very concept of selective permeation was looked upon with suspicion by many microbiologists, who believed that, in the absence of direct proof, it served mostly as a verbal "explanation" of certain results.

During the past few years, however, definite proof of the existence, in bacteria, of stereospecific<sup>2</sup> permeation systems, functionally specialized and distinct from metabolic enzymes, has been obtained. It now appears extremely likely that the entry into a given type of bacterial cell of most of the organic nitrilites which it is able to metabolize is, in fact, mediated by such specific permeation systems. None of these systems has been isolated or analyzed into its components. But the stereospecific component of certain of these systems has been indirectly identified as a protein, and defined by a combination of highly characteristic properties. The generic name "permeases" has been suggested for these systems. Although this designation may be criticized, it has the overwhelming advantage that its general meaning and scope are immediately understood.

The object of the present review is to discuss critically the recent evidence from different labo-

ratories concerning a few systems where the properties and identity of the stereospecific component can best be studied and where the physiological significance of "permeases" as connecting links between the intracellular and the external worlds is most clearly in evidence. We wish to emphasize that this is not a review of the literature on osmotic properties of bacteria, or on active transport. We shall be primarily interested in the specificity of permeation, and only secondarily in its thermodynamic aspects. Actually a certain amount of confusion has been entertained in this field because the question of the selectivity (stereospecificity) of permeation processes has not always been clearly distinguished from the problem of energetics of active transport of molecules across cellular membranes. Selective permeation need not necessarily be thermodynamically active. Conversely, active transport may be nonstereospecific. The fact that these two aspects of permeation processes are often, as we shall see, very closely associated renders the distinction even more important.

We shall therefore limit the discussion to the permeation of organic molecules, and exclude the problem of the penetration of inorganic ions such as phosphates about which the excellent review of Mitchell (55) may be consulted.

## II. ACCUMULATION, CRYPTICITY, AND SELECTIVE PERMEABILITY

That the entry of organic substrates into bacterial cells may be mediated by more or less selective permeation systems has been suggested primarily by two kinds of observations concerning, respectively: (a) the capacity of certain cells to accumulate internally certain nitrilites; (b) the state of "crypticity" of certain cells toward certain substrates, *i.e.*, their incapacity to metabolize a given substrate, even though they possess the relevant enzyme system.

Let us see why both accumulation and crypticity phenomena were strongly suggestive, yet inconclusive, as evidence of the operation of selective permeation systems.

The classical work of Gale on the uptake of

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<sup>2</sup> A stereospecific system is one whose activity is primarily dependent upon the spatial configuration of the reacting molecules.

amino acids in staphylococcal cells posed the problem of accumulation mechanisms 10 years ago (27, 28). As is well known, Gale and his associates found that staphylococcal cells grown on casein hydrolysate contain large amounts of glutamic acid, lysine, and other amino acids, which could be extracted by water from crushed, but not from intact, cells. These observations appeared to indicate that the cells were very highly impermeable to the amino acids. If this were true, then the entry of the amino acids could not occur by simple diffusion, since simple diffusion is by definition a reversible process: It had to be mediated by some special, unidirectional transfer mechanism. This conclusion was also suggested by the fact that glutamic acid enters the cells only in the presence of glucose. However, lysine, which is accumulated to a similar extent as glutamic acid, and is equally retained by intact cells, does not require glucose for its entry. An alternative mechanism therefore has to be considered, namely that the amino acids are retained within staphylococcal cells by intermolecular forces, for instance by some kind of macromolecular receptors. If so, no permeable barrier, nor any permeation mechanism need be assumed to account for the accumulation (28). As we shall see again later, these two alternative interpretations must both be considered and weighed against each other, whenever attempting to interpret the mechanism of accumulation of a compound by a cell. A choice between them is always difficult; all the more so since they are not mutually exclusive: proving a contribution to the accumulation process by one of these mechanisms does not in itself disprove contribution of the other.

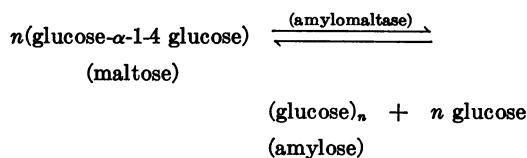
The paradoxical finding that enzymes active against a given substrate may, in some cases, be extracted from cells which, when intact, are inert toward the same substrate, has been noted many times and diversely interpreted by puzzled microbiologists. There is, of course, no paradox when the "cryptic" state of the cells concerns a whole class of chemical compounds, since there is no difficulty in assuming that the solubility and/or electrical properties of a class of compounds may forbid their passage through the cell membrane. The phosphorylated metabolites (nucleotides, hexose phosphates) provide classical examples.

The paradox arises when interpretations in terms of nonspecific forces or properties become inadequate; that is to say, when crypticity is

highly *stereospecific*. The study of the metabolism of disaccharides by yeasts has furnished several of the earliest described cases of specific crypticity. For instance, intact baker's yeast does not ferment maltose although autolyzates of the same yeasts contain maltase ( $\alpha$ -glucosidase). Analogous observations have been made with other yeasts for cellobiose, and cellobiase ( $\beta$ -glucosidase), sucrose and sucrase, etc. (52). Similarly, Deere *et al.* (19) described, in 1939, a strain of *Escherichia coli* which did not ferment lactose, although lactase ( $\beta$ -galactosidase) was present in dried preparations of these cells.

An essential point is that the cells which are cryptic towards a given carbohydrate, nevertheless behave as a rule quite normally towards other carbohydrates. For instance, an *E. coli* strain which is cryptic towards lactose metabolizes glucose, maltose, and other carbohydrates at a high rate. Now, if crypticity to a particular carbohydrate is attributed to the impermeability of the cell membrane, then the membrane must be impermeable to all compounds presenting similar solubility properties and molecular weight; that is to say virtually all carbohydrates. Therefore, those carbohydrates that do enter the cell and are metabolized at a high rate must be supposed to use some highly specific stratagem for getting through the barrier.

Several particularly striking examples of selective crypticity have been revealed by the studies of Doudoroff *et al.* (22-24). For instance, a mutant of *E. coli* was incapable of metabolizing glucose, although it metabolized maltose via the enzyme amyломaltase (66) which catalyzes the reversible reaction:



Although free glucose is liberated in this reaction, the organisms were found to metabolize quantitatively both moieties of the maltose molecule. Therefore, it appeared that glucose could be used when liberated intracellularly by amylo-maltase, while free glucose from the external medium could not be used by these cells. Later observations showed, moreover, that hexokinase could be extracted from these paradoxical organisms. The conclusion that the cells of this

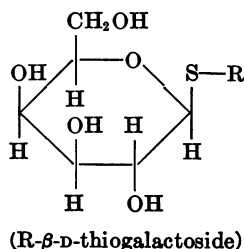
mutant strain were impermeable to glucose seemed inescapable. But a membrane impermeable to glucose could not possibly be permeable to maltose, except via a stereospecific permeation system.

This type of interpretation of specific crypticity effects, although quite logical, often appeared arbitrary and unreasonable since, to account for a metabolic paradox concerning a single compound, one had to assume the existence of a multitude of specific permeation systems for which no positive evidence existed, and towards which no direct experimental approach seemed open. An alternative interpretation was therefore often preferred; namely, that where specific crypticity occurred, it was due to a state of inactivity of the intracellular enzyme concerned. The activity was supposed to be released only upon release of the enzyme from the cell (5, 52, 62). This interpretation seemed simpler and more attractive in many respects than the specific permeation hypothesis for which only negative evidence could be adduced.

### III. GALACTOSIDE-PERMEASE

The actual demonstration and identification of a specific permeation system, as distinct from other similar systems and from intracellular metabolic enzymes, rests upon a sort of operational isolation *in vivo*, which requires a combination of different experimental approaches. The galactoside-permease system, which we shall now discuss, has offered remarkable opportunities in this respect (9, 60, 71).

Before introducing this system, it should be recalled that *Escherichia coli* metabolizes lactose and other galactosides via the inducible enzyme  $\beta$ -galactosidase. Analogs of  $\beta$ -galactosides where the oxygen atom of the glycosidic linkage is substituted by sulfur (34) are not split by galactosidase, nor are they used by *E. coli* as a source of energy, carbon, or sulfur (61, 38, 7, 60):



#### A. Accumulation of Galactosides in Induced *Escherichia coli*: Kinetics and Specificity

When a suspension of *E. coli*, previously induced by growth in the presence of a galactoside, is shaken for a few minutes with an  $\text{S}^{35}$  labeled thiogalactoside, and the cells are rapidly separated from the suspending fluid (either by centrifugation or by membrane filtration), they are found to retain an amount of radioactivity corresponding to an intracellular concentration of galactoside which may exceed by 100-fold or more its concentration in the external medium. Noninduced cells (*i.e.*, cells grown in the absence of a galactoside) do not accumulate any significant amounts of radioactivity.

The accumulated radioactivity is quantitatively extracted by boiling water. Chromatographic analysis of extracts shows a major spot, which by all criteria corresponds to the free unchanged thiogalactoside. A minor component (which may consist of an acetylated form of the galactoside) is also evident when accumulation has taken place in the presence of an external source of energy. This compound does not seem to be a product or an intermediate of the accumulation reaction, and its formation may be disregarded in discussing the kinetics of accumulation.

The accumulation is reversible: when the uptake of galactoside is followed as a function of time, a stable maximum is seen to be reached gradually (within 5 to 20 min at 34 C, depending on the galactoside used). If at this point an unlabeled galactoside is added to the medium at a suitable concentration, the radioactivity flows out of the cells (figure 1). The amount of intracellular galactoside at equilibrium, in presence of increasing external concentrations of galactoside,

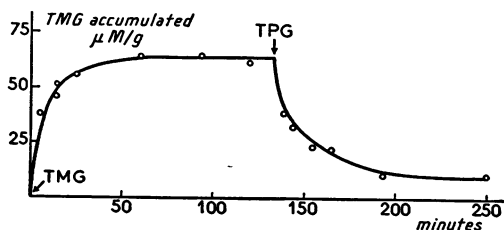


Figure 1. Accumulation of radioactive thiomethyl- $\beta$ -D-galactoside (TMG) by induced *Escherichia coli* at 0 C (71). At time indicated by arrow, addition of unlabeled thiophenyl- $\beta$ -D-galactoside (TPG).

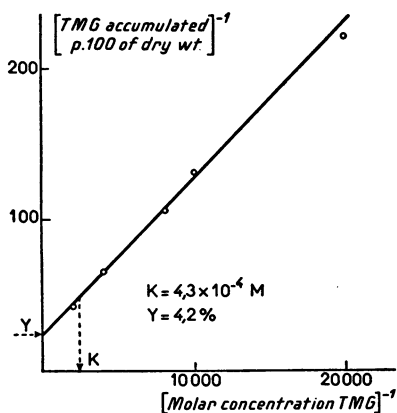


Figure 2. Accumulation of radioactive thio-methyl- $\beta$ -D-galactoside (TMG) as a function of external concentration (71). Reciprocal coordinates.  $Y$  and  $K$  are the constants of equation (1).

follows quite accurately an adsorption isotherm (figure 2). Calling the external concentration of galactoside  $G_{ex}$ , and the amount taken up by the cells at equilibrium  ${}^{eq}G_{in}$ , one may write:

$${}^{eq}G_{in} = Y \frac{G_{ex}}{G_{ex} + K} \quad (1)$$

$K$  is the dissociation constant of the "bacterium-galactoside complex," and  $Y$  is another constant, called capacity, which expresses the maximal amount of galactoside which the cells take up at saturating concentration of a given galactoside.<sup>3</sup>

The displacement of a labeled galactoside by another, unlabeled, galactoside also follows quite accurately the classical laws of competition for a common site (figure 3). This allows the determination of specific affinity constants for any competitive compound. The specificity of the system proves very strict: only those compounds which possess an unsubstituted galactosidic residue (in either  $\alpha$  or  $\beta$  linkage) (69) present detectable affinity for the competition site. Glucosides or other carbohydrates, even though they may differ from galactosides only by the position of a single hydroxyl, do not compete with the galactosides. Moreover, all the effective competitors which have been tested have proved also to be

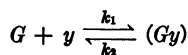
<sup>3</sup> The "total capacity" is defined as the capacity per unit volume of cell suspension. The "specific capacity" is the capacity per unit weight of organisms. It may be expressed in per cent dry weight or preferably in moles per unit dry weight.

accumulated within the cells. The affinity constant for each can then be determined either directly, by measurement of accumulation, or indirectly by displacement of another galactoside. The two values agree reasonably well.

The results show that the accumulation of galactosides within induced cells is due to, and limited by, stereospecific sites able to form a reversible complex with  $\alpha$  and  $\beta$  galactosides. However, a choice must be made between two entirely different interpretations of the role of these sites.

### B. Stoichiometric vs. Catalytic Model

The simplest interpretation (stoichiometric model) would be that the galactosides ( $G$ ) are accumulated within the cells in stoichiometric combination with specific receptor sites ( $y$ ), according to an equilibrium:



The constant  $K$  of equation (1) would then represent the dissociation constant of the complex  $Gy$ , while the constant  $y$  would correspond to the total number of available receptor-sites.

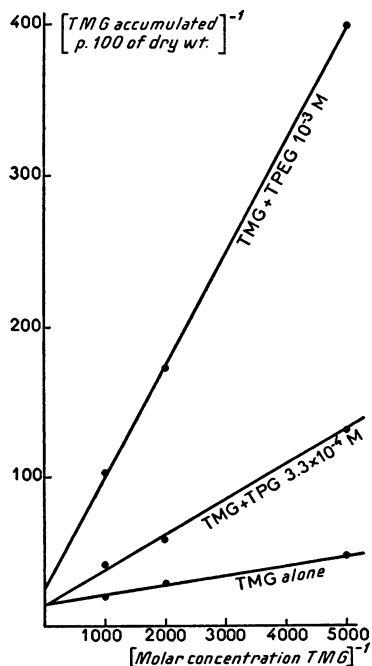
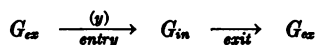


Figure 3. Competitive displacement of thio-methyl- $\beta$ -D-galactoside (TMG) by thiophenyl-ethyl- $\beta$ -D-galactoside (TPEG) and thiophenyl- $\beta$ -D-galactoside (TPG), in *Escherichia coli* (71).

The second interpretation (catalytic or permease model) assigns to the specific "permease" sites the role of *catalyzing* the accumulation of the galactosides into the cell, rather than serving as final acceptors. In order to account for the properties of the system, one is then led to the following scheme:



according to which the intracellular galactoside ( $G_{in}$ ) is a steady-state intermediate between an *entry* reaction, catalyzed by the stereospecific sites, and an independent *exit* reaction. The *entry* reaction involves the transitory formation of a specific complex between the sites and the galactoside, and should follow the kinetics of enzyme reactions. The *exit* reaction is assumed *not to involve the sites*, and its rate to be proportional to the amount of intracellular galactoside  $G_{in}$ .<sup>4</sup> According to these assumptions, the rate of increase of the intracellular galactoside is given by:

$$\frac{dG_{in}}{dt} = y \frac{G_{ex}}{G_{ex} + K} - cG_{in} \quad (2)$$

If  $\frac{y}{c} = Y$ , equation (2) reduces to equation (1)

for equilibrium conditions, when  $\frac{dG_{in}}{dt} = 0$ . The constant  $K$  again corresponds to the dissociation constant of the galactoside-site complex, while the capacity constant  $Y$  is now the ratio of the permease activity,  $y$ , to the exit rate constant,  $c$ . Insofar as the latter remains constant, the level of intracellular galactoside ( $G_{in}$ ) at equilibrium is proportional to the activity of the permease.

In choosing between the catalytic and the stoichiometric interpretations, the first argument to consider is one of common sense: in induced cells, the level of galactoside accumulation may be very high, and actually exceed 5 per cent of the dry weight of the cells. If the intracellular galactoside were adsorbed onto stereospecific sites (presumably associated with cellular proteins), there would have to exist, in highly induced cells, one such site for each fraction of cellular protein of molecular weight 2,000; an assumption which seems quite unreasonable.

The kinetics of intracellular galactoside accu-

<sup>4</sup> In principle, the rate of the exit reaction should probably be considered as proportional to the difference between  $G_{in}$  and  $G_{ex}$ . In practice,  $G_{ex}$  is negligible compared to  $G_{in}$ .

mulation also prove incompatible with the stoichiometric model while in good agreement with the assumptions of the permease model. The most significant facts in this respect are the following:

(a) According to the stoichiometric model, the rate of entry of galactosides should be proportional to the number of free sites and to the galactoside concentration. Actually, the initial rate of entry of a galactoside is not significantly faster than its rate of exchange during the steady state, even at saturating concentrations, when few sites remain free. Moreover, at saturating concentrations, the initial rate of entry is *independent* of galactoside concentration (figure 4) (41). Both findings are predicted by the permease model, according to which the initial and steady-state rates of entry should be equal, and proportional to the steady-state level of accumulation  $G_{in}$ . Also, in accordance with this expectation, the rate of entry at nonsaturating galactoside concentrations is proportional to the steady-state level. This means that the constant  $K$  of equation (1) corresponds effectively to the dissociation constant of the permease while the exit rate is proportional to  $G_{in}$ .

(b) According to the stoichiometric model, the capacity constant  $Y$ , i.e., the saturation value for intracellular accumulation, corresponds to the number of available specific sites and should be the same, with a given cell suspension, for all galactosides. Actually, the values vary rather widely from one to another compound, the ratio being, for example, 5 to 1 for thiomethyl-galac-

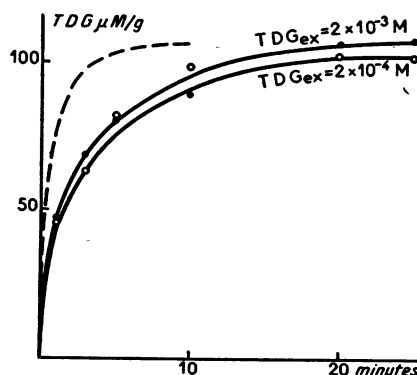


Figure 4. Uptake of radioactive thio-di- $\beta$ -D-galactoside (TDG) at two different saturating concentrations (41). It is seen that the initial rate of uptake is not significantly different at the two concentrations. The dotted line is the curve of the uptake at the highest concentration as expected on the basis of the stoichiometric model.

toside and thiophenyl-galactoside. This agrees with the catalytic model since both the activity constant of the permease, and the exit rate constant, should be expected to depend on the structure of the galactoside. Moreover, the capacity constant ( $Y$ ) and the affinity constant ( $1/K$ ) vary independently so that a galactoside endowed with relatively high affinity for the sites (*e.g.*, thio-di- $\beta$ -D-galactoside, or TDG) may be accumulated, at saturation, to a lesser extent than another one with lower affinity (*e.g.*, thiomethyl- $\beta$ -D-galactoside, or TMG). Therefore, according to the permease model, at suitable concentrations, more TMG molecules should be "displaced" from a cell by addition of TDG, than the cell takes up TDG molecules. Actually, in one experiment, addition of TDG ( $10^{-4}$  M), displaced 75 per cent of the TMG, *i.e.*, about 100  $\mu$ M/g for an uptake of 15  $\mu$ M/g of TDG.

Such results are evidently incompatible with the stoichiometric site hypothesis which may be dismissed. The kinetic evidence leaves no doubt that the role of the specific sites must be catalytic.

However, the specific purpose of the catalytic model which we have considered so far is to account for the fact that the steady-state level of intracellular accumulation is proportional to the activity of the permease. The model does not imply any specific assumption regarding the nature of the forces which bind (loosely) the "internal" galactoside to the cell, thereby allowing its accumulation. Again two different interpretations of such a model might be considered. According to one, the cell membrane would be freely permeable to galactosides. The "accumulated" galactoside therefore could not be free. It would be bound loosely to some nondiffusible cell constituent. The *entry* reaction would then consist of the catalytically activated binding of the galactoside to the  $x$  constituent, while the *exit* reaction would involve the dissociation of the  $G$ - $x$  complex:



The second interpretation (permease model, *sensu stricto*) assigns the binding essentially to a high degree of impermeability of the cell membrane (or other osmotic barrier) toward carbohydrates. The permease sites catalyzing the *entry* must then be assumed to be associated with the osmotic barrier itself. The simplest interpretation

of the *exit* reaction then is to consider it as "leakage" through the membrane, increasing in rate as the internal concentration builds up, to the point where it equilibrates the intake.<sup>5</sup>

It should be stressed that the kinetics of galactoside accumulation do not, by themselves, allow a choice between these two different interpretations. The first one is unlikely, however, for the same common sense reasons as the stoichiometric model: the amounts of galactoside accumulated in certain cells are so high that it would be difficult to find enough molecules or groups of any kind to account for the binding. However, the most decisive reason for adopting the second interpretation is the evidence that cells genetically or otherwise devoid of permease are *specifically cryptic toward galactosides*. This evidence will be reviewed later (see page 176).

### C. Metabolic and Energy Relationships of the Permease Reaction

Even adopting the permease model as valid, it would be rash to consider the intracellular galactoside as necessarily free and in solution in a phase comparable to the external medium. The physical state of the intracellular galactoside being undetermined, the work involved in the accumulation process is unknown. That the accumulation process must involve work and that the necessary metabolic energy must be channeled via the permease system itself is evident, however, from the fact that the steady-state concentration is proportional to the rate of the permease reaction. For it were supposed that the accumulation process released, rather than consumed, energy or that another system, independent of the permease, channeled the energy for accumulation, then the equilibrium concentration would be independent of permease activity, although the *rate of entry* might remain proportional to it.

This conclusion is confirmed by direct evidence

<sup>5</sup> It may be useful to point out that caution should always be exercised in interpreting differences of intracellular accumulation at equilibrium as due to effects on the entry reaction. It is possible if not probable that certain conditions may affect the exit reaction and thereby alter the equilibrium, by influencing, for example, the properties of the cell membrane. Direct measurements of the rates of entry and exit are required to decide such an issue.

indicating that the accumulation of galactoside by the permease is linked to the metabolic activity of the cell.

In the first place, the accumulation process is inhibited by typical uncoupling agents such as 2,4-dinitrophenol ( $M/250$ ) or azide ( $M/50$ ) (9, 71). When these inhibitors are added in the steady state, the intracellular concentration decreases rapidly. An external source of energy is not required, however, although the system is somewhat more active when one is present.

In addition, it should be mentioned that, according to Kepes (40), a small, but significant, increase of respiratory activity occurs when a suitable thiogalactoside is added to cells possessing permease, while noninduced cells or cells genetically devoid of permease show no such increase. The increase is so small that it cannot be detected in the presence of an external source of energy, when the oxygen consumption is too intense. It is only observed as an increase of the endogenous respiration. This extra oxygen consumption is accompanied by an extra  $CO_2$  production. The extra  $CO_2$  produced in the presence of unlabeled thiogalactoside, by cells previously homogeneously labeled with  $C^{14}$ , is also labeled, showing that the extra oxidation corresponds to an extra consumption of endogenous reserves, not to an oxidation of the galactoside itself. This extra oxygen consumption could correspond to the work involved in concentrating the galactosides into the cells (40).<sup>6</sup>

There is, at present, no available evidence concerning the mechanism of the energy coupling. Special attention should be called to the following point: while the uncouplers  $NaN_3$  or 2,4-DNP inhibit the accumulation of galactosides, they do not inhibit to a comparable extent the *in vivo* hydrolysis of galactosides by intracellular galactosidase. Since, as we shall see later, there is little doubt that the permease limits, *in vivo*, the rate of this hydrolysis, it would seem that the uncouplers do not inhibit the entry of galactosides via the permease, but only the energy coupling which allows the permease reaction to function as a pump, against a concentration gradient. When the concentration gradient is in favor of

entry, which is so when the intracellular hydrolase splits the substrate as soon as it enters, the uncouplers appear to exert no inhibitory action.

#### D. The Induced Synthesis of Galactoside-Permease. Permease as Protein

The fact that galactoside-permease is an inducible system has been of particular value for its study and characterization. As we have mentioned, the system is active only in cells previously grown in the presence of a compound possessing a free unsubstituted galactosidic residue. No other carbohydrates show any inductive activity. Not even all galactosides are inducers. The specificity of induction can best be studied using thiogalactosides which are not hydrolyzed or "transgalactosidated" in the cells. The specificity pattern of induction is strikingly parallel to that of  $\beta$ -galactosidase although there are some minor differences, which may be significant (table 1), in the relative inducing activity of different compounds. Since probably all galactosides are concentrated by the permease, all inducers are also "substrates" of the system. However, several compounds known to be actively concen-

TABLE 1\*

Induction of galactoside-permease and  $\beta$ -galactosidase by various thiogalactosides

Inducer Added during Growth	Galactoside-Permease (Specific Activity), $\mu$ moles TMG/g	$\beta$ -Galactosidase (Specific Activity), $\mu$ moles ONPG Hydrolyzed $\times \text{min}^{-1} \times \text{mg}^{-1}$
None.....	<2	2
Methyl- $\beta$ -D-galactoside.....	176	8,500
Propyl- $\beta$ -D-galactoside.....	181	8,500
Isopropyl- $\beta$ -D-galactoside....	124	9,500
Hexyl- $\beta$ -D-galactoside.....	14	8.5
Phenyl- $\beta$ -D-galactoside.....	5	2
Benzyl- $\beta$ -D-galactoside.....	10	16
Phenyl-ethyl- $\beta$ -D-galactoside	59	110
Galactoside- $\beta$ -D-thiogalactoside.....	10	4

The cultures were made on synthetic medium, in presence of inducer  $10^{-3}$  M. The permease was measured in presence of radioactive  $10^{-3}$  M TMG.

\* From H. V. Rickenberg, G. N. Cohen, G. Buttin and J. Monod (71).

<sup>6</sup> It remains to be seen whether it may not be linked, in part at least, with the formation of the "minor component" which was mentioned on page 171.

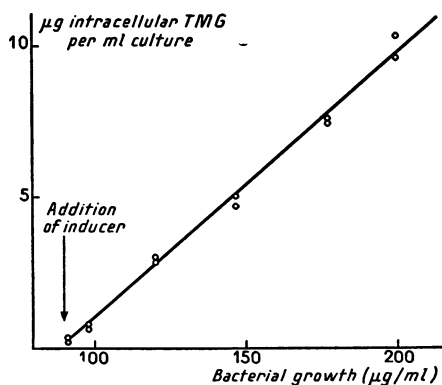


Figure 5. Induced synthesis of galactoside-permease in growing *Escherichia coli* (71). Carbon source: succinate. Inducer: thiomethyl- $\beta$ -D-galactoside (TMG)  $5 \cdot 10^{-4}$  M. It is seen that the increase in total permease activity is proportional to the increase of bacterial mass from the time of addition of inducer.

trated (phenyl- $\beta$ -D-thiogalactoside; thio-di- $\beta$ -D-galactoside) show little or no inducing activity.

The induction is effective only under conditions allowing the synthesis of protein; it is blocked by chloromycetin or in the absence of a required amino acid. Perhaps more significant yet is the fact that the system is not formed in the presence of  $\beta$ -2-thienylalanine. This compound, it should be recalled, does not inhibit the synthesis of protein in *Escherichia coli* but it is incorporated into the proteins formed in its presence which are biologically inactive (67).

Thus, there is little doubt that the induction corresponds in effect to the synthesis of the specific protein component of the system. The kinetics of this induced synthesis follow a remarkably simple law. As measured by the total capacity, the permease increases linearly with the total cell mass, from the time of addition of the inducer (figure 5). One may write:

$$Y = p\Delta x$$

where  $x$  is the increase in cell mass after addition of inducer, and  $p$  is the differential rate of synthesis (65). This relation, it should be recalled, is typical of inducible enzymes (studied under adequate conditions) and suggests that the increase in capacity, i.e., in permease, corresponds to the *de novo* synthesis of a protein (38, 65, 63). It is of interest to mention that, while the induction results in an increase of capacity, i.e., permease activity, it has no effect on the affinity constant

( $1/K$ ). These findings confirm that the interpretation of the two constants is correct.

If it is added that the induction of permease, like that of many enzymes, is blocked by glucose (cf. M. Cohn, in this issue), it will be seen that the inductive behavior of this system is in every way similar to that of the most typical inducible enzymes. Taken together with the evidence concerning the kinetics and specificity of accumulation, these findings leave no doubt that a specific, inducible, protein is responsible for the activity of this system. The complete system may, and probably does, involve also noninducible and nonspecific, or less specific, components. The term "permease" should be used primarily to designate the specific, inducible, protein component of the system, while the expression "permease system" implies all the components.

It is not excluded, of course, that the system may comprise a sequence of two (or more) inducible proteins catalyzing successive steps in the entry reaction. There is, at present, no necessity for this assumption.

A further characteristic of the permease may be mentioned at this point: it is an SH-dependent system, inhibited by *p*-chloro-mercuribenzoate (pCMB). The inhibition is partially reversed by cysteine and glutathione. Substrates, i.e., thiogalactosides, protect against this inhibition in proportion to their affinity, showing that the mercurial acts directly on the specific, galactoside-binding, protein component of the system. (It may be added that  $\beta$ -galactosidase is also rapidly inactivated by pCMB, and also protected by galactosides.)

#### *E. Functional Significance of Galactoside-Permease; Specific Crypticity*

As we have already stressed, the kinetics of galactoside accumulation do not, by themselves, impose the permease hypothesis. The only assumption which is required to account for the kinetics of accumulation is that the stereospecific sites act catalytically. This could be described, as we indicated, by a model which would not involve any permeability barrier. It is only by studying the relationship of the accumulation system to other systems, in particular,  $\beta$ -galactosidase, that we can decide between the permease and other models. As a matter of fact, one of the problems to consider is whether the permease and the hydrolase really are distinct sys-

tems, rather than two functions of the same system.

If the permease model is correct—i.e., if (a) the cells are virtually impermeable to galactosides, except for the specific activity of the permease, (b) the permease is distinct from  $\beta$ -galactosidase, (c) galactosidase is strictly intracellular—one should expect at least two phenotypes among mutants of *E. coli* incapable of metabolizing galactosides, one type corresponding to the loss of the permease, the other to the loss of the galactosidase. Actually, the two predicted types have been found, together with a few others which we shall discuss later, among spontaneous and induced "lactose" mutants of *E. coli*.

Cells of the first mutant type ("absolute-negatives"), grown in presence of a suitable inducer (thiomethyl-galactoside), accumulate normal amounts of galactoside, but they form no detectable trace of  $\beta$ -galactosidase. These organisms will, in particular, accumulate large amounts (up to 20 per cent dry weight) of lactose, while the induced, normal cells do not accumulate lactose, which is split and metabolized as soon as it is taken up.

Cells of the second mutant type (cryptics), grown in presence of sufficiently high concentrations of thiomethyl-galactoside, form normal amounts of galactosidase (as revealed by extrac-

tion), but none or only traces, of permease. So long as they are physiologically intact, these cells hydrolyze galactosides at a much slower rate than normal cells possessing equal amounts of galactosidase. Moreover, the rate of hydrolysis is a linear function of galactoside concentration instead of being hyperbolic, as in the normal type (figure 6) (36). This indicates that the rate of hydrolysis of galactosides in these cells is limited by a diffusion process rather than by a catalyst. The organisms are, in particular, almost inert towards lactose, while their metabolic behavior towards other carbohydrates is normal. In other words, these organisms are specifically cryptic towards galactosides.

These properties of the cryptic mutants, which for a long time had appeared paradoxical, as we recalled (page 170), are immediately explained if  $\beta$ -galactosidase is effectively inside a highly impermeable barrier which the galactosides can cross only by forming a complex with the permease. The existence of these two mutant types proves that permease and  $\beta$ -galactosidase are genetically and functionally distinct, and that they normally form a metabolic sequence *in vivo*.

The study of the hydrolysis of galactosides *in vivo*, in wild-type organisms, also brings out the functional role of the permease.

For instance, the hydrolysis *in vivo* of true galactosides is inhibited by thiogalactosides, al-

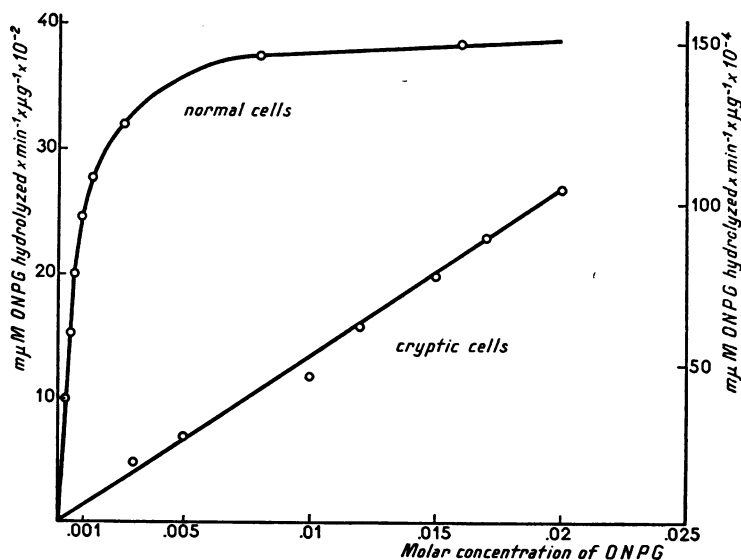


Figure 6. *In vivo* hydrolysis of ortho-nitro-phenyl- $\beta$ -D-galactoside (ONPG) by *Escherichia coli* (36). Upper curve: normal type. Lower curve: cryptic (permeaseless) mutant. Ordinates on left apply to upper curve. Ordinates on right apply to lower curve.

TABLE 2

*In vivo* hydrolysis of *o*-nitrophenyl- $\beta$ -D-galactoside by cells in which the syntheses of  $\beta$ -galactosidase and permease have been differentially inhibited

	$\beta$ -Galactosidase (units/ml Culture), $\mu$ m- moles ONPG Hydrolyzed $\times \text{min}^{-1} \times \text{ml}^{-1}$	Galactoside-Permease (units/ml Culture), $\mu$ m-moles TMG Accumulated/ml	Rate of <i>in Vivo</i> Hydrolysis, $\mu$ m-moles ONPG Hydrolyzed $\times \text{min}^{-1} \times \text{ml}^{-1}$
Cells grown without <i>p</i> -fluorophenylalanine..	636	24	84.9
Cells grown with <i>p</i> -fluorophenylalanine....	660	3.8	9.8
Per cent reduction of activity by growth in presence of the analog.....	0	84	88

Two cultures of *Escherichia coli* ML30 were grown respectively in the absence and in the presence of DL-*p*-fluorophenylalanine  $5 \times 10^{-4}$  M. Thiomethyl- $\beta$ -D-galactoside  $5 \times 10^{-4}$  M was added 1 minute after the analog. The cell mass was allowed to increase in the two cultures from 63  $\mu$ g dry weight to 252  $\mu$ g dry weight/ml. The activities of extracted  $\beta$ -galactosidase, of the galactoside-permease, the rate of *in vivo* hydrolysis of ONPG were determined according to (71).

lowing a determination of the affinities of the inhibitors for the total system. The pattern of affinities which is thus disclosed is quite different from that of  $\beta$ -galactosidase studied *in vitro*, but it is very close to the pattern of affinities of the permease, suggesting that, in normal induced wild type, the permease, rather than the galactosidase, is the limiting factor for the hydrolysis of galactosides. This is probably also the explanation of the fact, which has been known for several years, that the *in vivo* hydrolysis of certain galactosides by the wild type is slower than expected on the basis of the extractable galactosidase activity of the cells (49).<sup>7</sup>

A striking experiment allows the conversion of normal cells into phenocopies of the cryptic, permeaseless organisms. It is based on the fact that cells grown in the presence of *p*-fluorophenylalanine incorporate the analog into their proteins, in place of tyrosine and phenylalanine (67). Now it would seem that certain proteins formed under these conditions retain their specific activity, while others do not. Actually, when normal *E. coli* is grown and induced in presence of suitable concentrations of *p*-fluorophenylalanine, the cells form normal amounts of  $\beta$ -galactosidase, but only traces of permease. These cells behave like cryptics, in that they hydrolyze *o*-nitrophenylgalactoside at a much lower rate than controls possessing equal amounts of galactosidase and higher levels of permease (table 2).

All these experiments, therefore, concur in

<sup>7</sup> This conclusion does not necessarily imply that the *in vivo* and the extracted  $\beta$ -galactosidase must have necessarily the same activity.

demonstrating that the permease controls *in vivo* the communications between the intracellular  $\beta$ -galactosidase and the external medium.

#### *F. Functional Relationships of Permease: Induction*

The control by permease of the entry of galactosides into the cells carries other consequences which are particularly interesting. Galactosides are not only substrates (or specific inhibitors) of permease and galactosidase; they are also (7, 61, 71) inducers of the two systems. Therefore, by controlling the intracellular concentration of inducers, the permease should control the kinetics of  $\beta$ -galactosidase and of its own induction. As this problem is reviewed in this same issue by M. Cohn (pp. 140-168), we shall only briefly record here the main points.

First, the induction of  $\beta$ -galactosidase requires much higher concentrations of inducers in cryptic mutants than it does in the wild type; actually, with thiomethyl-galactoside, more than a 100-fold difference in external inducer concentration is required for saturation of the induction system. Since the wild type effects a 100-fold or more intracellular concentration of the inducer while the cryptic does not concentrate it at all, the interpretation is immediate.

Second, at low inducer concentrations, the kinetics of galactosidase synthesis, expressed in differential rates (increase of enzyme *versus* increase in bacterial mass), is autocatalytic while it is linear at higher (saturating) concentrations. This is easily understood since, at low (nonsaturating) concentration, the differential rate of synthesis will depend on the intracellular concen-

tration factor, *i.e.*, on the specific activity of the permease, and therefore it should increase as induction proceeds.

If this interpretation is correct, the cryptic mutants, devoid of permease, should show linear kinetics of induction at all concentrations of inducer. This actually occurs as shown in figure 7. This result, obtained by Herzenberg (36), is an important confirmation of the contention (contrary to frequently expressed views) that the induced synthesis of enzymes does not involve an increased synthesis of enzyme-forming system.

At low concentrations of inducer, however, the induction of the permease-galactosidase system is autocatalytic, since permease is a system which concentrates its own inducer. This entails some remarkable consequences. *E. coli* cells which already possess permease are induced by very low concentrations of inducer, which are ineffective on noninduced cells. As the permease is distributed between daughter cells, these will inherit this "sensitiveness" and go on producing enzyme in presence of these low inducer concentrations. Therefore, under suitable conditions, the capacity to synthesize permease may be manifested as a self-perpetuating and as a clonally distributed property (13; M. Cohn, this issue; 68). The interest of this effect as a possible model of certain types of cellular differentiation is evident.

#### G. Genetic Relationships of Galactosidase and Galactoside-Permease

Permease and galactosidase are genetically distinct, as we already noted, since they are affected by different specific mutations. However, there are interesting genetic relationships between the two systems.

In the first place, a fairly large number of mutations manifested by the loss of the capacity to synthesize either  $\beta$ -galactosidase or galactoside-permease have been isolated in *E. coli* K12 and they have been found to be all very closely linked, although no two of them were alleles (J. Lederberg, unpublished; Monod and Jacob, unpublished). (Actually, the complexity of the locus in question had been recognized several years ago by E. Lederberg (48)). This finding is interesting, although not very surprising, since the work of Hartman, Demerec, and others (20) has revealed that the genes controlling enzyme sequences in bacteria are often, if not as a rule, closely linked, and may even be arranged in the same order as the reactions themselves.

Although most mutations affect specifically either permease or galactosidase, giving rise to the "cryptic" and "absolute negatives" mentioned previously, a few appear to suppress both systems simultaneously. These might be deletions. But another mutant type deserves special mention. This is the constitutive (12, 50) in which *both* the permease and the  $\beta$ -galactosidase are formed in the absence of inducer (71). The mutation to constitutive is spontaneous and may occur in "cryptic" organisms devoid of permease. These "constitutive cryptics," which form very large amounts of  $\beta$ -galactosidase without external inducer but are unable to metabolize lactose, may in turn mutate spontaneously to a permease-positive type able to grow on lactose. When this occurs, the permease also is constitutive. Therefore, this single step, spontaneous mutation to constitutive, controls the constitutive *versus* inducible character of *both* galactosidase and permease. It should be added that the locus of the

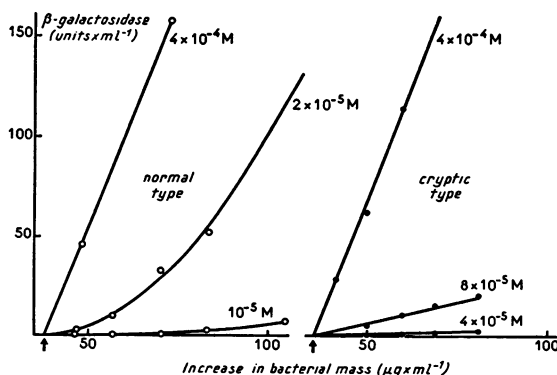


Figure 7. Kinetics of induced  $\beta$ -galactosidase synthesis in normal *Escherichia coli* and in the cryptic mutant type (36). Inducer: iso-propyl-thio- $\beta$ -D-galactoside.

TABLE 3\*  
*Glucuronide-permease of Escherichia coli*

Bacterial Suspension	Additions	S <sup>35</sup> -TPU Accumulated	Accumulation of Induced Control
		μM/g	%
Noninduced.....	None	0	0
Induced by methyl-β-glucuronide 10 <sup>-3</sup> M.....	None	47	100
Same.....	Phenyl-β-glucuronide 2 × 10 <sup>-2</sup> M	0	0
Same.....	Thiomethyl-β-glucuronide 2 × 10 <sup>-2</sup> M	6	13
Same.....	Thiomethyl-β-glucuronide, methyl ester 2 × 10 <sup>-2</sup> M	47	100
Same.....	Methyl-β-glucoside 2 × 10 <sup>-2</sup> M	46	97

Bacterial suspensions were shaken at 37 C for 10 minutes with S<sup>35</sup>-thiophenylglucuronide (TPU) 5 × 10<sup>-4</sup> M.  
\* From F. Stoeber (77).

TABLE 4  
*Independent synthesis and independent functioning of galactoside-permease and glucuronide-permease*

Cells Previously Grown with Succinate as Main Carbon Source, in Presence of:	Permease Activities (Accumulation of Radioactive Compound, μM/g) in Presence of Following Assay Mixtures:			
	S <sup>35</sup> thio-galacto-side*	S <sup>35</sup> thio-glucur-onide†	S <sup>35</sup> thio-galacto-side + S <sup>35</sup> thioglu-curo-nide	S <sup>35</sup> thio-galacto-side + S <sup>35</sup> thioglucur-onide
Isopropyl-β-D-galactoside.....	93	1	124	—
Methyl-β-D-glucuronide.....	1	57	—	56
Glucuronide + galactoside.....	100	64	85	66

\* S<sup>35</sup> labeled thiomethyl-β-D-galactoside.  
† S<sup>35</sup> labeled thiophenyl-β-D-glucuronide.

constitutive mutation is also closely linked to the loci controlling specifically the capacity to synthesize β-galactosidase and permease.

IV. OTHER CARBOHYDRATE PERMEASES OF *ESCHERICHIA COLI*

The existence, in *Escherichia coli*, of a specific permease which controls the metabolic utilization of galactosides, e.g., lactose, implies, almost necessarily, as we have already pointed out, that other equally specific permeation systems must control the permeation of the numerous other carbohydrates which *E. coli* is normally able to metabolize. However, the positive identification

of a permease requires an experimental material (in particular, adequate nonmetabolizable radioactive substrates) which is not always readily available. So far, only two other carbohydrate permeation systems for specific carbohydrates have been identified in *E. coli*, one active on glucosides, the other on glucuronides.

A. *Glucuronide-Permease*

Stoeber (77) has recently described an inducible system which concentrates glucuronides in *E. coli*. It should be recalled that *E. coli* forms an inducible β-glucuronidase. Stoeber found that S<sup>35</sup>-labeled thiophenyl-β-D-glucuronide (35), which is not hydrolyzed by glucuronidase, is accumulated, unchanged by glucuronide-induced *E. coli*. The accumulation, which is inhibited by 2,4-DNP and NaN<sub>3</sub>, is reversible, and the intracellular *versus* extracellular concentration follows an adsorption isotherm [equation (1) with *K* ca. 10<sup>-4</sup> M]. The system is strictly stereospecific: the accumulation is competitively inhibited by free, unsubstituted glucuronides, not by glucosides, galactosides or other carbohydrates (table 3). The induced formation of the system is blocked by β-2-thienylalanine.

By all these properties, this system is closely analogous to galactoside-permease, while it is sharply defined by its strict specificity of combination and of induction. The two systems may be either separately or simultaneously induced in the same strain of *E. coli*. In the doubly induced cells, glucuronide and galactoside are simultaneously accumulated, each to a similar extent as in the singly induced cells (table 4).

The two permeases are thus synthesized independently, and function independently, without any cross interference.

*B. Glucoside Accumulation in  
Escherichia coli* (64)

*E. coli* does not measurably metabolize  $\alpha$ -methyl-glucoside. However, suspensions of *E. coli* shaken with  $C^{14}$  labeled  $\alpha$ -methyl-glucoside accumulate, reversibly, up to 100  $\mu$ moles/g of the compound. Radioautograms of hot water extracts from the cells show a single spot with the same  $R_f$  as free  $\alpha$ -methyl-glucoside. The accumulation is not inhibited significantly by  $NaN_3$  or 2,4-DNP. It is reversible and the variation of internal versus external concentration of  $\alpha$ -methyl-glucoside follows again an adsorption isotherm [equation (1) with a  $K$  of  $2 \times 10^{-4}$  M].

The system is constitutive: cells grown in presence of glucose or succinate, with or without  $\alpha$ -methyl-glucoside, are about equally active.

The specificity of the system has not been studied in detail, for lack of adequate compounds. However, the accumulation of  $\alpha$ -methyl-glucoside is very powerfully inhibited by glucose, even at extremely low concentrations. There is little or no inhibition with fructose and galactose. Since glucose is used by the cells, it is difficult to determine whether the inhibition is competitive and whether it is due to glucose itself or to a product of its metabolism.

These observations suggest that the accumulation of  $\alpha$ -methyl-glucoside in *E. coli* is due to a constitutive permease system possessing a high affinity for glucose. We have already recalled Doudoroff's studies on a mutant of *E. coli* K12 which appeared to be cryptic towards glucose (see page 170). As it turns out, cells of this glucose-negative strain also lack the capacity to accumulate  $\alpha$ -methyl-glucoside although, after proper induction, they prove capable of accumulating galactosides. Moreover, 12 independent glucose-positive isolates, obtained from the glucose-negative mutant by transduction with an adequate phage (P1), were found to have also regained the capacity to accumulate  $\alpha$ -methyl-glucoside.

These findings indicate that the  $\alpha$ -methyl-glucoside accumulating system must be closely related to the system presumably responsible for the permeation of glucose in *E. coli*. Further studies are required to identify these systems.

V. THE PERMEATION OF KREBS CYCLE INTER-  
MEDIATES AND OTHER ORGANIC ACIDS IN  
PSEUDOMONAS AND AEROBACTER

The operation of the permeation systems which we have studied so far can be tested directly, independently of the activity of the corresponding metabolic enzymes. The existence, in *Pseudomonas* and *Aerobacter*, of specific systems insuring the permeation of certain intermediates of the Krebs cycle and of other organic acids has been inferred from a different kind of evidence, essentially from crypticity relationships.

A. Citrate and other Intermediates of the  
Krebs Cycle

The interferences of permeability effects in the metabolism of intermediates of the Krebs cycle had been frequently suspected in the past, as an explanation of discrepancies observed between the metabolism of intact cells and the enzymic activities of extracts (25, 39, 78).

In 1953, Kogut and Podoski (43), and Barrett *et al.* (2) discovered independently that the oxidation of citrate and of other intermediates of the cycle by intact cells of *Pseudomonas* was adaptive, while the enzymes of the cycle itself were constitutive. The specificity of this inductive effect, as shown by Kogut's results (table 5), reveals the existence of at least five different inducible systems. It is particularly interesting to note, for instance, that cells grown on citrate, and able to use citrate without lag, are still cryptic towards *iso*-citrate and *cis*-aconitate. This illustrates strikingly the specificity of the systems involved. In sharp contrast to the strictly adaptive behavior of intact cells, extracts from cells grown on citrate, fumarate or succinate showed no significant difference in their oxidative activity towards citrate (2) and other intermediates of the cycle (43).

Both groups of authors formulated the hypothesis that the adaptive behavior of intact cells was due to specific, inducible permeation factors. They tested the effects of agents known to inhibit the synthesis of active enzymes. Barrett *et al.* (2), in particular, showed that the amino acid analogs, ethionine and *p*-fluorophenylalanine, blocked adaptation to citrate. Thus unadapted *Pseudomonas* cells are cryptic towards most intermediates of the Krebs cycle. Adaptation to a given intermediate suppresses crypticity towards the corresponding compound, and this

TABLE 5\*

*Oxidation of tricarboxylic acid cycle intermediates by washed suspensions of Pseudomonas sp.*

Growth Substrate	Substrate for Oxidation									
	Succinate	Fumarate	Malate	Oxalacetate	Pyruvate	Acetate	Citrate	cis-Aconitate	iso-Citrate	$\alpha$ -Ketoglutarate
Succinate.....	+	+	+	+	0	0	0	0	0	0
Fumarate.....	+	+	+	+			0			0
Malate.....	+	+	+	+			0			0
Acetate.....	0	0			+	+	0			0
Citrate.....	0	0	+		0	0	+	0	0	0
iso-Citrate.....	0	0	+				+	+	+	0
$\alpha$ -Ketoglutarate.....	0	0			0	0	0			+

+ = Substrate oxidized linearly from the moment of tipping.

0 = Substrate not linearly oxidized initially.

\* From M. Kogut and E. P. Podoski (43).

"suppression of crypticity" requires protein synthesis.

An entirely similar situation has since been discovered, in *Aerobacter*, for citrate utilization, by Green and Davis (18), who showed, in addition, that the adaptation was inhibited by glucose. As we have already recalled, this "glucose effect" is typical of many, in fact of most, inducible enzymes.

Gilvarg and Davis (29) had previously found a mutant of *Escherichia coli* which lacked the condensing enzyme while it possessed all the other enzymes of the Krebs cycle. This mutant required  $\alpha$ -ketoglutarate for growth, but it could not utilize citrate in its place. In contrast, a mutant of *Aerobacter*, which also lacked the condensing enzyme, grew equally well on either  $\alpha$ -ketoglutarate or citrate. These findings show clearly that citrate is an obligatory intermediate of  $\alpha$ -ketoglutarate synthesis, in both organisms, but that *E. coli* is, for all practical purposes, impermeable to it, while *Aerobacter* can be "decrypted" by proper adaptation. It may be recalled, at this point, that the operation of a "complete" Krebs cycle in microorganisms had been repeatedly denied, precisely because many microbes, such as yeast and *E. coli*, did not metabolize certain intermediates of the cycle. It is now established beyond doubt, not only that the enzymes of the cycle exist, but that the cycle actually operates *in vivo*, in *E. coli* as well as in yeast (29, 44, 72, 76, 79, 80).

The findings summarized previously leave little doubt that the entry of Krebs cycle intermediates into bacteria is controlled by stereo-

specific permeases and that the failure of certain organisms to metabolize certain intermediates is due to their incapacity to synthesize the appropriate permease system. It is likely that the actual operation of these systems could be tested directly if proper analogs of their substrates were available, or if proper mutants were used.

#### *B. Tartaric Acid Permeation in Pseudomonas*

Recent observations of Shilo and Stanier (75) have given clear indications of the existence of stereospecific permeation factors for different isomers of tartaric acid in *Pseudomonas*. The attack of each isomer by most strains is due to a distinct inducible dehydrase which produces oxalacetic acid (45, 47, 74). Two kinds of observations showed that, besides the specific dehydrase, an additional, inducible factor is required for the *in vivo* metabolism of tartrates.

In the first place, when cells were grown on limiting amounts of tartrate as sole source of carbon and energy, depletion of tartrate caused the population to enter the stationary phase, invariably followed by a loss of the capacity for immediate attack on tartrate. This loss could not be explained by the inactivation of any of the known intracellular enzyme systems involved in the dissimilation of tartrate, including in particular the specific dehydrase, as tested with extracts.

The dissimilated cells, in other words, were cryptic towards tartrate while they still metabolized oxalacetate. The oxidative activity of the dissimilated cells could be regained rapidly by re-exposure to tartrate. But UV irradiation

blocked this readaptation, although it had no effect on the activity of the system, suggesting that readaptation involved resynthesis of a protein component which had presumably been inactivated during the dissimilation period.

Further indications that the access of tartaric acid isomers to the intracellular dehydrases is controlled by strictly stereospecific factors were given by the study of the inhibition *in vitro* and *in vivo*. *Meso*-tartaric acid is a powerful inhibitor of the *d*-dehydrase, and *d*-tartaric acid is a powerful inhibitor of *meso*-dehydrase in extracts; however, no such inhibition is observed in whole cells adapted to a single isomer. But if one uses cells able to attack the *three* isomers of tartrate, the oxidation of *meso*-tartrate can be inhibited *in vivo* by *d*-tartrate.

These facts could perhaps, like most specific crypticity effects, be explained by *ad hoc* alterations of the enzymes upon release from the cell. It is much more probable that they reveal a marked difference of stereospecific requirements, between the intracellular enzyme and a specific permeation factor. In fact, the results suggest that the permeation factors for tartrate isomers may be more exacting, in their stereospecificity, than the metabolic enzymes. The nature of the isomerism of tartaric acids makes this problem particularly intriguing. It may well be that the marvelous discriminative power possessed by the microorganisms which, about 100 years ago, helped Pasteur (70) to separate the different isomers of tartaric acid, was based largely on the properties of their permeases.

## VI. AMINO ACID PERMEASES

### A. The Accumulation of Exogenous Amino Acids by *Escherichia coli*

Many instances have been reported in the past in which the growth of auxotrophic mutants requiring amino acids was inhibited competitively by other structurally related amino acids; whereas the nonexacting wild-type was not inhibited (1, 21, 37, 42). To explain such data, various schemes were proposed, some of which (42) assumed a selective permeability barrier where the interactions were supposed to occur. However, no positive evidence was given in favor of this hypothesis.

This problem can best be studied by using labeled compounds to follow the uptake of amino acids by the cells (10, 11). In particular, the

uptake of radioactive valine by *Escherichia coli* K12 has been studied in some detail. It was found that when *E. coli* K12 is shaken at 37 C, in presence of this amino acid, under conditions where protein synthesis was blocked, radioactivity was rapidly accumulated into the cells, in amounts corresponding to a concentration factor (with respect to the external medium) of up to 500.

Chromatographic analysis of the radioactive material extracted by boiling water shows that the concentrated material consists exclusively of valine. The amounts of intracellular valine vary with the external concentration, according to an adsorption isotherm; the "capacity" (see page 172) of the system at saturating external concentration ( $5 \times 10^{-5}$  M L-valine) is of the order of 20  $\mu$ moles per g dry weight, i.e.,  $4 \times 10^6$  molecules of L-valine per bacterial cell. The apparent dissociation constant of the system is of the order of  $3 \times 10^{-6}$  M.

The accumulation is inhibited by 2,4-DNP and NaN<sub>3</sub>, and is optimal in the presence of an external energy source.

The accumulation of valine is reversible: the intracellular C<sup>14</sup>-valine can be displaced by non-radioactive valine, and also by the structurally related amino acids, leucine or isoleucine, while phenylalanine or proline, even at much higher concentrations, have shown no effects. Moreover, only the L-isomers are effective competitors. Substitution of the amino or carboxyl groups of the competitors, or replacement of the isopropyl group of valine by a dibutyl, diphenyl or dibenzyl group, suppresses all competitive capacity. Similarly, peptides containing valine, leucine, or isoleucine have little or no affinity for the valine accumulating system (table 6). The system responsible for accumulation is therefore strictly stereospecific. A quantitative study of the displacement shows that the antagonistic action of leucine or isoleucine is competitive (table 7).

This system is constitutive: it is present in organisms grown in the absence of valine or of any other amino acids. The total valine accumulating capacity of a growing culture increases linearly with the bacterial mass; this increase is inhibited by 5-methyl-tryptophane or thienylalanine, which do not stop protein synthesis, but are known to inhibit the synthesis of active enzymes (67).

The existence of several other systems similar to the valine one, but different in specificity, has been demonstrated in *E. coli*. One system is char-

TABLE 6\*

Structural conditions required for the competitive displacement

Additions	Internal Accumulation of L-Valine
M	$\mu$ moles/g
None.....	15.7†
L-isoleucine $5 \times 10^{-5}$ .....	2.8
L-leucine $5 \times 10^{-5}$ .....	2.1
Unlabeled L-valine $10^{-3}$ .....	2.1
D-valine $10^{-3}$ .....	19.2
D-isoleucine $10^{-3}$ .....	17.2
D-leucine $10^{-3}$ .....	12.5
DL-N-monomethylvaline $10^{-3}$ .....	18.3
DL-valinamide $10^{-3}$ .....	15.9
DL-dibutylalanine $10^{-3}$ .....	12.5
DL-diphenylalanine $10^{-3}$ .....	19.1
DL-dibenzylalanine $10^{-3}$ .....	18.5

Radioactive DL-valine was present in all suspensions (5 millimicromoles L-valine/ml). A sample was taken after 1-minute incubation at 37 C; then the presumed competitor was added and a new sample was taken after 1 minute.

\* From G. N. Cohen and H. V. Rickenberg (11).

† The controls without addition of each experiment differ at maximum of  $\pm 7\%$  from this mean value.

acterized by the capacity to accumulate L-phenylalanine. Radioactive phenylalanine is displaced by nonradioactive L-phenylalanine, but not by the D-isomer; it is also displaced by *p*-fluorophenylalanine, but not by phenyl-lactate, phenylpyruvate or phenylserine, nor by unrelated amino acids such as isoleucine or proline.

Another system accumulates L-methionine, which is displaced by nonradioactive L-methionine or by L-norleucine, but not by the D-isomers, nor by phenylalanine or proline or other unrelated amino acids.

The three systems studied are thus stereospecific and independent one from the other, as is proved by the absence of any effect of the typical substrate of each upon the functioning of the other two.

Britten, Roberts, and French (6), using different techniques, independently found that various exogenous amino acids were accumulated by *E. coli*, independently of protein synthesis. L-proline, in particular, is highly concentrated and the results suggest that the proline concentrating system is specific for this one amino acid.

TABLE 7\*

Competitive displacement of radioactive L-valine by L-isoleucine

Radioactive L-Valine	L-Isoleucine	Internal Accumulation of L-Valine
M	M	$\mu$ moles/g
$5 \times 10^{-6}$	0	19.1
	$2.5 \times 10^{-6}$	7.8
	$5 \times 10^{-6}$	3.1
$5 \times 10^{-6}$	0	34.1
	$10^{-5}$	30.4
	$5 \times 10^{-5}$	19.0
	$10^{-4}$	8.3
	$5 \times 10^{-4}$	5.5

\* From G. N. Cohen and H. V. Rickenberg (11).

Thus, except for inducibility, the amino acid concentrating systems are very similar to the galactoside concentrating system for the interpretation of which we have already discussed the catalytic *versus* stoichiometric models. We have seen that the evidence eliminates the latter in favor of the permease theory. However, the analogy between the two classes of systems would be insufficient to decide in favor of one or the other model. The validity of the permease model for the amino acid systems must be discussed on the basis of direct evidence.

To begin with, we may remark that the "common sense argument" based on the amount of substrate accumulated is valid for amino acids as well as for galactosides. For instance, proline may, according to Bolton *et al.* (4), be accumulated to the extent of several hundred  $\mu$ moles per gram, a quantity roughly equivalent to  $\frac{1}{3}$  the number of ribonucleic acid (RNA) nucleotides and  $\frac{1}{20}$  the total number of protein bound amino acids in the cell. Such a result would be very difficult to interpret on the hypothesis that the retention of the amino acids is due to binding to nondiffusible molecules.

#### B. Physiological Interactions between Amino Acids Explained by the Permease Model

The strongest arguments which favor the permease theory for amino acids is that it explains most satisfactorily the so far poorly understood physiological interactions observed in *E. coli* between structurally related amino acids.

We shall first consider valine and its effects on the growth of different strains of *E. coli*:

a. *Toxic effects of valine on E. coli K12.* It is known that the growth of *E. coli* K12 is inhibited by valine and that the inhibition is released by the addition of isoleucine (81) or leucine (73). The D-amino acids show no action either as inhibitors or as antagonists.

If the valine accumulating system is, in effect, a permease system which controls not only the accumulation of valine, but actually the entry of this amino acid into the cell and its access to all intracellular systems, the antagonism is immediately accounted for; the ratio isoleucine/valine necessary to displace 50 per cent of the valine is the same as the ratio of isoleucine/valine that restores 50 per cent of the normal growth. The detoxifying effect of isoleucine and leucine is thus undoubtedly linked to the inhibition of valine accumulation by the permease. The toxic effect of valine is therefore conditioned by the activity of the permease, but it is not an inherent consequence of it, since a valine-resistant mutant possesses a valine permease of the same affinity and activity. Consequently, the toxic effect of valine need not have anything to do with the intracellular metabolism and/or synthesis of isoleucine and leucine. Actually, it has been shown that the toxicity of valine for *E. coli* K12 is due to an alteration of the proteins synthesized in its presence (8).

b. *The growth of auxotrophic mutants of E. coli.* The growth of auxotrophic mutants of *E. coli* requiring one of the three amino acids: valine, leucine, or isoleucine is competitively inhibited by the two other members of the group. Here again, the ratio of isoleucine/valine at which the growth of a valine-requiring mutant is inhibited by 50 per cent, corresponds to the 50 per cent displacement ratio for valine accumulation through the permease in presence of isoleucine. The interpretation of these effects is again simple and straightforward in terms of the permease model while interpretations in terms of reciprocal effects upon biosynthetic pathways (37) were complex and unsatisfactory.

c. *Incorporation of exogenous valine into proteins by wild-type of Escherichia coli.* In normal wild-type *E. coli* (K12 excepted) neither leucine, isoleucine, nor valine exerts any positive or negative effect on the growth rate. It is, however, possible to demonstrate that the incorporation of exogenous valine is controlled by a system which

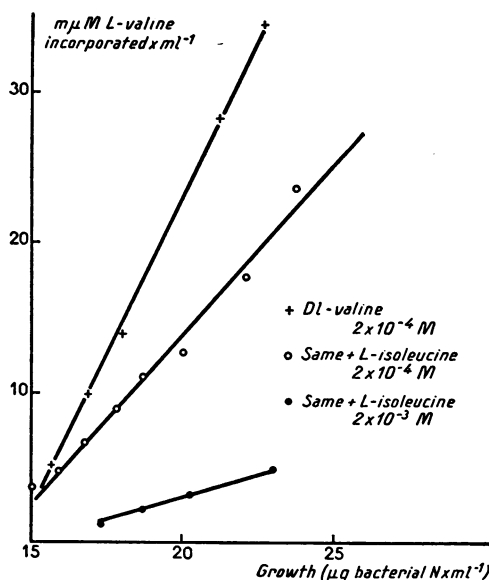


Figure 8. Incorporation of radioactive exogenous L-valine by wild-type *Escherichia coli* in absence and in presence of unlabeled L-isoleucine, at increasing concentrations (see text, this page) (11).

has the specificity of the permease. When wild-type *E. coli* ML is grown in presence of radioactive valine, this exogenous amino acid competes very efficiently with the valine which is endogenously synthesized from the carbon source of the medium: 95 per cent of the valine incorporated into the protein is radioactive. Under these conditions, the total radioactivity incorporated increases linearly with the increase in bacterial mass during growth. The slope of the straight line obtained measures the specific radioactivity of valine in the proteins synthesized from the time of addition and gives therefore the ratio of exogenous to endogenous valine in the newly formed proteins.

As is seen in figure 8, when the cultures are grown in presence of radioactive valine and unlabeled isoleucine, the specific activity of the proteins decreases as the concentration of isoleucine increases. Since, as we have mentioned, the presence or absence of valine or isoleucine is without action on the growth of these cells, this effect could hardly be attributed to a drastic alteration of the valine content of the proteins. It is evidently due to an altered ratio of incorporation of endogenous to exogenous valine into the proteins. Again, this effect is explained and predicted by the permease model. We can thus give

TABLE 8\*  
*Interactions between valine, leucine and isoleucine in Escherichia coli*

Organism	Effect of Valine on Growth	Antagonist	Effect of Antagonist on Accumulation of Valine	Effect of Antagonist on Growth
K12S	Inhibits growth	Isoleucine, leucine	Competitive displacement	Suppresses growth inhibition
ML328f	Required for growth	Isoleucine, leucine	Competitive displacement	Inhibits growth
Valine-resistant mutant of K12S or <i>E. coli</i> ML	None	Isoleucine	Competitive displacement	Nil. Inhibits incorporation of exogenous, but not of endogenous, valine

\* From G. N. Cohen and H. V. Rickenberg (11).

a unitary explanation of the valine-isoleucine antagonism for *E. coli* K12, for the auxotrophs, and for the normal *E. coli* strains. The three apparently unrelated phenomena now clearly appear to be different expressions of the same initial event; namely, competition of structurally related amino acids for a structure which has the same specificity and affinity characteristics as the valine-accumulation system. All the evidence concurs in showing that, while this structure is essential for the entry of exogenous amino acids into the metabolic space of the cells, it plays no role in the synthesis or incorporation of internally synthesized amino acids. Table 8 summarizes the information for the various types of strains.

Many other examples of inhibition of the utilization of a given amino acid by another structurally related natural amino acid, or by structural analogs, have been described in the literature. Lampen and Jones (46), and Harding and Shive (33) have described the inhibition of the growth of *E. coli* by norleucine and its competitive reversal by methionine. The first authors conclude from the competitive aspect of the antagonism that norleucine inhibits utilization, rather than synthesis, of methionine. Harding and Shive apply the methods of inhibition analysis and draw the conclusion that methionine functions in the biosynthesis of leucine, isoleucine, and valine, probably in their amination, and that norleucine inhibits this function of methionine. The progress made since 1948 in the study of the biosynthesis of valine, leucine, and isoleucine by *E. coli* has revealed no such function of methionine. Actually, it has been found that norleucine is incorporated in the proteins of *E. coli* where it substitutes for methionine (R. Munier and

G. N. Cohen, unpublished); however, the synthesis of methionine is unimpaired, in the presence of norleucine, and the nonincorporated methionine is found in the culture medium. The known properties and specificity of the methionine permease account completely for the competitive suppression of norleucine inhibition by methionine.

A similar situation is encountered with thienylalanine and *p*-fluorophenylalanine which also cause the synthesis of "false proteins." The analogues are incorporated in place of phenylalanine and tyrosine in the proteins of *E. coli*, and the inhibition of growth which ensues is due to the biological inactivity of these "false proteins."

Suppression of the inhibition by phenylalanine is again explained by the directly determined displacements at the permease level. Other interpretations, based on *p*-fluorophenylalanine inhibiting the synthesis of tyrosine from phenylalanine (3) are erroneous, since such a pathway for tyrosine synthesis has been excluded in *E. coli* (17).

An antagonism which has been correctly analyzed occurs in the inhibition of the growth of *E. coli* by diamines, studied by Mandelstam (53). The wild type of *E. coli* is not inhibited by diamines, whereas the growth of a lysine-requiring mutant is inhibited by the C<sub>6</sub>, C<sub>8</sub> and C<sub>7</sub> diamines. Direct estimation has shown that the inhibition of growth is due to the inhibition of uptake of lysine.

The essential justification of the permease model for the amino acid accumulation systems in *E. coli* is that it accounts for the observed antagonisms. The stoichiometric model or variations of it could possibly account for some of

these effects, but only at the expense of additional *ad hoc* assumptions. The number and the variety of the effects which are simultaneously explained by the permease model leave no doubt that it must be valid in its essential features.

This rapid review of some examples of antagonisms shows how cautious one should be in the interpretation of antimetabolic activities of structural analogs. The possibility that the effects observed are due to specific permeases must be taken into account. However, it would probably be equally dangerous to use the permease interpretations indiscriminately in cases where no actual data concerning the specificity and relative affinities of the presumed systems are available.

#### C. Accumulation of Amino Acids by Other Microorganisms

As mentioned before, the experiments of Gale and his co-workers (27, 28) provided the first example of the accumulation of amino acids by bacterial cells. It is also well known that yeasts possess a "free amino acid pool" and may accumulate exogenous amino acids into this pool (32). An essential difference between these gram-positive organisms and *E. coli* is that the "free" intracellular amino acids are not in equilibrium with amino acids in the external medium. It seems probable, nevertheless, that the bulk of the amino acids are indeed free, which implies necessarily the existence of an impermeable barrier and the occurrence of active transport (28). Whether this active transport is mediated by stereospecific factors comparable to *E. coli* permeases is uncertain. Halvorson and Cohen (31) have observed that the rate of uptake of valine and phenylalanine into the pool of yeast is inhibited by structurally unrelated amino acids, including D-isomers. A similar situation had been disclosed previously in *Neurospora crassa* by Mathieson and Catchside (54), who showed that the uptake of histidine in this organism is inhibited by a whole series of other amino acids. It should be stressed that these authors were able, on this basis, to explain the fact that histidine-requiring mutants of *Neurospora* are inhibited by other amino acids while the wild type is uninhibited.

#### GENERAL DISCUSSION

As an economical way of summarizing the more general conclusions which appear justified by the

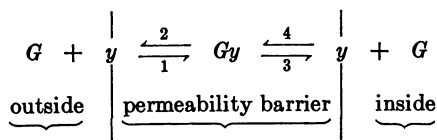
evidence reviewed here, it will be convenient to consider a model cell to which we shall try to attribute only the *minimal* properties required to account for the behavior of the different permeation systems studied. The discussion of this model will also give us an occasion of bringing in certain elements of evidence which we have not considered so far, because they did not pertain directly to the study of any one permeation system.

In attempting to construct such a model it might be useful to consider the information concerning selective permeation of organic compounds in tissues of higher organisms. Actually, the wealth of information is so considerable and so complex that summarizing it in a few statements is virtually impossible. It must be noted, however, that by far the largest fraction of this information concerns "transtissular" permeation, that is to say, transport from one extracellular space to another extracellular space across more or less complex cellular tissues or organs. The problem is experimentally quite different from the one we have attempted to analyze in microorganisms. Yet, Wilbrandt (84) has shown that the more likely interpretation of such "transtissular" permeation is a "membrane carrier mechanism," involving specific carriers, operating within otherwise impermeable cellular membranes.

The most significant observations directly concerning cellular permeation in cells of higher organisms have been made on erythrocytes (51, 84). The existence, in the erythrocytes of primates, of a transmembrane carrier system for hexoses, is clearly established. The system appears to function exclusively in equilibrating intracellular and extracellular concentrations; there is no evidence of active transport. Since, in this work, the rates of hexose permeation are determined by measurements of osmotic equilibration, there is no ambiguity as to the state of the intracellular compound, and to the existence of a real osmotic barrier. The intervention of a specific carrier is shown by the nonlinearity of the rates of entry with respect to concentrations, and by competition between different sugars. The stereospecificity of the carrier is not very strict, since several different hexoses and pentoses appear to compete for the same system. The existence in these cells of different independent carrier systems for different compounds or classes of compounds is likely, but they do not seem to have been clearly identified one from the other.

Let us now describe the model which we shall use as a basis of discussion.

The bacterial cell is supposed to be enclosed within an osmotic barrier (55) highly impermeable toward polar substances, such as carbohydrates, hydroxy and other organic acids, amino acids, and the like. The impermeability of the barrier is not supposed to be absolute; however, leakage in either direction may occur, slowly tending to equilibrate the inside and outside concentrations. In places within the barrier, there exist different proteins (the permeases) which are able to form stereospecific, reversible complexes with different hydrophilic compounds. Dissociation and association of the specific complex may occur either on the inside or on the outside of the osmotic barrier. The effect of a permease, therefore, is to activate catalytically the equilibration of the concentrations (activities) of the substrate on either side of the membrane, *i.e.*, inside and outside the cell, according to the scheme:



Many permease systems, but not necessarily all of them, are coupled to an energy donor, the net effect of which is to inhibit the "inside" association reaction (reaction 4). When this occurs, the substrate accumulates within the impermeable barrier, *i.e.*, within the cell, until the difference of internal and external concentrations is high enough for the nonspecific leakage through the permeability barrier to equilibrate the entry via the permease.

Let us now consider the justification, the possible meanings, and the limitations of the different assumptions contained in this scheme. The first essential assumption is the existence of an osmotic barrier enclosing the whole cell and impermeable to polar compounds. The justification for this assumption is that it accounts at once (a) for the capacity of bacterial cells to accumulate and retain certain compounds in an apparently free state; (b) for the crypticity of certain cells towards certain polar compounds. Both effects have been amply documented here and we have discussed the reasons which make other interpretations of accumulation (see pages 173-174) and of specific crypticity (see page 176) virtually impossible. We need only to refer to discussion.

As final evidence of the existence of a cellular

osmotic barrier in bacteria, we cite the recent work of Mitchell and Moyle (58), which allows the estimation of the actual intracellular osmotic pressures within certain bacterial cells. Using an elegant technique, they find a pressure of 20-25 atmospheres for *Staphylococcus aureus*, (*Micrococcus pyogenes* var. *aureus*) a value which indicates that most of the low molecular weight intracellular compounds must be free and in solution within the osmotic barrier (59). Mitchell and Moyle have also devised (57) new techniques for studying the rate of equilibration of internal and external osmotic pressure of bacterial cells. Using various carbohydrates and polyhydric alcohols, they find that, in general, these rates are extremely low; sometimes so low that osmotic equilibrium is never reached.

These observations illustrate the low permeability of bacterial cells toward sugars in general. However, they are not altogether free of complexities and apparent contradictions, and they cannot, we believe, be interpreted directly in terms of *rates of permeation*, since most of the compounds used are metabolized by the cells at high, variable, and unknown rates. It would be of great interest to see these techniques applied to unnatural isomers, which would not be metabolized, and for which specific permeases would presumably not be available. The tests performed with such unnatural compounds would give an estimation of the truly *nonspecific* permeability of the membrane.

We shall not dwell on the important problem of the physical nature of the osmotic barrier. Modern work with "protoplasts" indicates clearly that the cell wall, while responsible for the resistance of the cells to internal hydrostatic pressure (83) is not a significant element of the permeability barrier since the permeability properties of protoplasts are similar to those of intact cells. Worthy of mention here is that galactoside-permease is present and functional in *E. coli* protoplasts, as prepared by Rickenberg (personal communication). The existence of a separable membrane, limiting the protoplast (83) is indicated by the formation of "ghosts" when protoplasts burst. According to Mitchell and Moyle (56), "membrane" fractions of *Staphylococcus aureus* have a high lipid content, which may account for the impermeability of the cell to hydrophilic compounds. These facts certainly encourage the identification of this "ghost" structure with the cellular osmotic barrier and

bearer of the permease proteins, but the evidence for this identification is, so far, purely circumstantial.

Before turning to another problem, note that the existence of several independent subcellular osmotic units with different permeability characteristics is not excluded, and may have to be considered in the future. For the time being, there is no necessity for this more complex picture.

The second essential assumption of the model is the existence of different, independent, stereospecific permease proteins, functionally specialized for the permeation of specific compounds and distinct from the intracellular metabolic enzymes dealing with the same compounds. The presentation of the evidence bearing on this point has been the object of the present review. We need not discuss this evidence again, but it will be useful to summarize briefly as follows the essential experimental justifications of the permease hypothesis:

1. Certain strains or mutants of bacteria are specifically cryptic, *i.e.*, metabolically inert towards a given compound, although possessing a competent intracellular enzyme system for the metabolism of the compound, and although capable of metabolizing other closely related compounds at a high rate. This suggests that the permeation of such compounds into the cells involves a stereospecific process.

2. Certain polar compounds are (reversibly) accumulated by certain cells, and the accumulation is inhibited by steric analogs of the compound. Conversely, sterically different compounds are accumulated simultaneously without any cross-interference.

These observations show the dependence of accumulation on a stereospecific component and the functional independence of different accumulation systems.

3. The kinetics of accumulation prove that the stereospecific sites act as intermediates, not as final acceptors for the accumulated compound. Moreover, the blocking or inactivation of the stereospecific component results in the cells becoming specifically cryptic towards the corresponding compound.

Therefore, the specific sites act as catalysts for the entry of the compounds into the metabolic space of the cell.

4. The formation of the stereospecific component of certain systems is provoked by specific

inducers. This induced formation is blocked by agents which prevent selectively the synthesis of biologically active proteins. These observations show that the specific components of different systems are different proteins individually and independently synthesized by the cell.

5. The capacity of the cell to synthesize a given permeation system may be suppressed by specific mutations which have no effect on the corresponding intracellular metabolic enzyme. Other specific mutations, which suppress the intracellular enzyme, do not suppress the corresponding permeation system. Similarly, certain agents inhibit the synthesis of the permeation system, without interfering with the synthesis of the corresponding metabolic enzyme. These findings prove that the permeation systems are distinct and independent of the homologous metabolic enzymes.

At the present time, at most eight different permeases have been positively identified in a single organism (*E. coli*). This is not a large number. However, as we have stressed several times, proof of the existence of a permease, and of specific crypticity relationships, for a single compound belonging to a homogeneous class, necessarily implies that other, equally specific, permeases insure the permeation of the other compounds of the class which are rapidly metabolized by the same cells. On this basis, it can be estimated that *E. coli* must possess, or be able to synthesize, at least 30 to 50 different permeases dealing with organic substrates. The entry of certain inorganic ions, in particular phosphate, is undoubtedly catalyzed, as it has been shown by the work of Mitchell (55). However, the participation of permeases, in the sense defined above, cannot be tested in this process since none of the necessary criteria (stereospecificity, independence from intracellular enzyme, specific crypticity) is applicable.

The third assumption is that the permease may be coupled to an energy-yielding reaction, and thereby act as a pump, or uncoupled and thermodynamically passive, when it functions as an equilibrator of outside and inside concentrations. This is a synthetic and abstract description of the observed relationships, rather than an assumption. The justification for assuming a coupling is, of course, in part, the effect of metabolic inhibitors. But it should be pointed out that this evidence is not so unequivocal as is generally believed. The metabolic "uncouplers" may act

in different ways; they are inactive with certain systems (glucoside-permease) where work is probably performed. The effect, or absence of effect, of an external source of energy is not a good test either, since such a source is often not required. Finally, the best evidence that work is performed in the accumulation of galactosides in *E. coli* is kinetic. As we have seen (see p. 174), this evidence shows that the coupling must be at the level of the specific permeation reaction itself.

There is no reason, however, to suppose that coupling is necessary for the permease protein to act as a thermodynamically passive permeation catalyst. Moreover, as we pointed out, the uncouplers seem to inhibit only the *accumulation*, not the permeation of galactosides. Although the evidence on this last point is certainly insufficient, it is of interest in suggesting a unified interpretation of specific permeation mechanisms, whether or not active transport is performed.

In the minimal model which we have set up, no attempt has been made to represent the actual mechanism of energy coupling. It should be noted, however, that the coupling necessarily implies the participation of further components in the system, namely coenzymes or transporters of chemical potential. Even when the permease acts passively, the participation of other (non-specific) components may be required. Obviously the actual mechanism of specific permeation must be more complex than the deliberately bare and abstract model we have set up. The object of this model has been to symbolize only the indispensable assumptions, and the identified components: the permeability barrier, the stereospecific permease protein, the dependence of permeation on the formation of a reversible permease-substrate complex, the possibility for the system to perform work or not, depending on conditions. Of the existence and nature of nonspecific components, of the detailed mechanism of action of the permease, and of the mechanism of coupling, nothing positive can be said at present. Such problems have given rise to very ingenious speculations (15, 30, 82) into which we shall not go because a whole variety of equally likely, albeit quite different, schemes would have to be considered.

We may, however, briefly consider the question of whether the permeases are proper enzymes or not, and show that it is not meaningless. As we have seen, the permeases behave exactly like enzymes in many characteristic respects.

They are proteins which form stereospecific, reversible complexes with certain compounds; certain of them are inducible under the same conditions as typical inducible enzymes; galactoside-permease is controlled by a system of mutations exactly parallel to the system which controls  $\beta$ -galactosidase.

However, enzymes are characterized essentially by the property of catalyzing a *chemical reaction* of their substrates, *i.e.*, the formation, or rupture, or transfer of covalent bonds. There is, at present, no direct evidence that such events occur at the substrate level, with permeases. When there is work performed in the process, it is virtually certain that covalent bonds are broken or transferred and, to that extent, the permease acts like an enzyme in activating their breakage or transfer. But these chemical events need not involve the permeating substrate itself. Whether or not the permeation process involves a *chemically altered* form of the substrate as an intermediate, is one of the first problems which will have to be considered in studying the mechanism of action of the permeases.

The hypothesis, that the rapid permeation of hydrophilic organic compounds into cells is insured by stereospecific and functionally specialized protein components of the plasma membrane, is not new or original. It has been frequently invoked in the past to account for selective permeability effects, the most precise and elaborate formulations having been given by Danielli (14-16). The recent microbiological work summarized in this review has given precise and extensive experimental support to this concept. The specific inducibility and independent mutability of certain of the bacterial permeases, allow us to individualize, identify, and study these systems under exceptionally favorable conditions.

The examples we have reviewed leave little doubt that the entry of all the main organic nutrilites (carbohydrates, organic acids, amino acids) into bacteria is controlled by specific permeases.

Thus the role of permeases as chemical connecting links between the external world and the intracellular metabolic world appears to be decisive. Enzymes are the element of choice, the Maxwell demons which channel metabolites and chemical potential into synthesis, growth and eventually cellular multiplication. Occurring first in this sequence of chemical decisions, the

permeases assume a unique importance; not only do they control the functioning of intracellular enzymes, but also, eventually, their induced synthesis. Moreover, since the pattern of intermediary metabolism appears more and more to be fundamentally similar in all cells, the characteristic, differential, chemical properties of different cells should depend largely on the properties of their permeases.

#### ADDENDUM

Recent experiments performed by Sistrom at the Pasteur Institute answer the critical question whether galactosides intracellularly accumulated by the action of galactoside permease in *Escherichia coli* are free or bound (see page 174). These experiments depend on the fact that protoplasts of *E. coli* retain the permeability properties of intact cells, including in particular galactoside permease activity (see pages 188-189), while their resistance to osmotic pressure differences is very greatly reduced. Differences in osmotic pressure between the intra- and extracellular phases may result from either a decrease of the external osmolality or an increase of the internal pressure resulting, for example, from intracellular accumulation of a compound in an osmotically active state, i.e., in a free state. The bursting of protoplasts is easily measured as a decrease of the optical density of the suspension, so that protoplasts can be used as sensitive indicators of variations of their own osmotic pressure. By comparing the extent of lysis which occurs when the internal pressure increases as a result of permease activity with that caused by a known decrease in external pressure, one can determine approximately the intracellular concentration of free permease substrate at the time of lysis.

Using this experimental principle, Sistrom studied the lysis caused by accumulation of various galactosides in *E. coli* protoplasts prepared by a modification of the lysozyme-versene technique of Repaske [Biochim. et Biophys. Acta, **22**, 189-191 (1956)]. The strain used was a mutant possessing an inducible galactoside permease, and devoid of galactosidase, i.e., able to accumulate, but unable to hydrolyze lactose or other galactosides. It was observed that protoplasts prepared from induced cells (i.e., cells grown in presence of an inducer of galactoside permease) and suspended in 0.1 M phosphate buffer, underwent lysis upon addition of M/100

lactose, while protoplasts from uninduced cells were insensitive to addition of lactose. Comparisons with lysis provoked by decreasing the molarity of the suspending buffer showed that the induced protoplasts had accumulated free lactose to the extent of about 22% of their dry weight, a figure closely approximating direct estimations performed on intact cells. The addition of a rapidly metabolizable carbohydrate, namely glucose, did not result in any significant lysis.

These experiments demonstrate that the bulk, if not the totality, of the substrates of galactoside-permease are accumulated within the cells in a free form. Therefore the accumulating mechanism must be catalytic, and the retention is due to a permeability barrier, not to binding or other intermolecular forces.

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